CYTOCHROME *c* BINDS TO LIPID DOMAINS IN ARRAYS OF MITOCHONDRIAL OUTER MEMBRANE CHANNELS

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ABSTRACT Computer-averaged electron microscopic images of negatively stained crystalline arrays of fungal mitochondrial outer-membrane channels in the presence and absence of cytochrome *c* were compared. Neither the apo- nor the holo- forms of cytochrome *c* significantly changed the stain distribution in the protein regions of the channel arrays. However, both forms of cytochrome *c* caused significant stain exclusion from the lipid domains in the arrays, suggesting binding of the polypeptides at these loci. The implications of binding of apocytochrome *c* to clusters of exposed phospholipids on the mitochondrial outer membrane are discussed with respect to the mechanism of uptake of this polypeptide by mitochondria.

INTRODUCTION

Only a small fraction of the protein components of the mitochondrion are synthesized within the organelle itself. The majority are encoded by nuclear DNA and are synthesized on free cytoplasmic ribosomes (1, 2). Current models for import of these proteins into the mitochondrion have as an obligatory first step the attachment of the polypeptides to the cytoplasmic surface of the mitochondrion. Subsequent uptake of the polypeptides often involves processing of the polypeptides (i.e., proteolytic cleavage of terminal residues), the nature of which depends to some extent on the ultimate destination of the polypeptide within the mitochondrion (3–5).

The nature of the initial attachment of import polypeptides to the mitochondrial surface is currently unknown. In the case of yeast, fungal, and rat mitochondria, there is considerable evidence for the existence on the mitochondrial outer membrane of specific, saturable receptors for particular import polypeptides (6–9). In general, mild proteolysis of the mitochondrial surface abolishes binding of precursors, suggesting that the receptors are protein in nature. However, direct identification and/or isolation of the putative receptor proteins has not as yet been achieved.

The binding of apocytochrome *c* to the outer membrane surface of *Neurospora* mitochondria has been characterized by Hennig et al. (7). Binding is optimal under conditions of low ionic strength and near-neutral pH. Apocytochromes *c* from other eukaryotes (yeast, plants, and animals) displace *Neurospora* apocytochrome *c* from *Neurospora* mitochondria. However, pretreatment of mitochondria with holocytochrome *c* or polylysine does not prevent subsequent binding of the apocytochrome, suggesting specific recognition of apocytochromes and not general affinity for polycations. Scatchard plots of the binding of *Neurospora* apocytochrome *c* to *Neurospora* mitochondria are nonlinear, and can be fit by assuming two classes of binding sites, with affinity constants differing by a factor of 5 (2.2×10^7 and 4×10^6 M^{-1}). Hennig et al. concluded that the higher affinity binding sites "represent mitochondrial receptors involved in posttranslational transfer of apocytochrome *c* into mitochondria" (7).

The mitochondrial concentration of high affinity receptors for apocytochrome *c* deduced from the Scatchard plots in the above-mentioned study is large, 90 pmol/mg mitochondrial protein or ~600 per μm^2 of mitochondrial surface. As Teintze and Neupert later noted (10), the only outer membrane polypeptide known to be present at this concentration is the 31-kD channel-forming protein called VDAC, for voltage-dependent, anion-selective channel, or mitochondrial porin (11, 12). Thus, VDAC is a leading candidate for the role of primary protein receptor in the mechanism of apocytochrome *c* uptake by mitochondria.

Our laboratory is engaged in study of the structure of the channels formed by VDAC in the mitochondrial outer membrane (13–15). In particular, we have used computer processing of electron microscopic images to characterize the structure of the planar crystalline arrays that the channel protein forms in the *Neurospora* membrane. When negatively stained, the arrays are dominated by stain-accumulating sites, which have been shown (by three-dimensional reconstruction from tilt-series projection data) to be stain-filled cylinders that span the 5–6-nm-thick membrane (16). Since the stains used are very hydrophilic, the stain-filled cylinders probably represent the aqueous channels formed by VDAC in phospholipid bilayers. The projected diameter of these cylinders is

2–2.5 nm (15, 17), consistent with the inner diameter of the VDAC channel inferred from electrical step conductance measurements (12). Recent image analysis of electron micrographs of unstained VDAC arrays embedded in amorphous ice has delineated the protein and lipid domains within the membrane arrays (15).

To test the hypothesis that the pore protein VDAC might be a receptor for apocytochrome *c*, we devised the following experiment. The approach is the same as that recently applied to the localization of toxin-binding sites on acetylcholine receptors (18). Projected electron microscopic image averages of the VDAC channel array in different negative stains have been well characterized (17). In uranyl acetate, the channels appear as approximately circular, dense stain sites, 4.5–5 nm apart, which are rimmed by smaller stain minima (Fig. 1 *a*). The channels occur in clusters of six, which are flanked by regions of intermediate staining density. These lightly stained regions correspond to lipid (lowest density) in maps of frozen-hydrated VDAC arrays (15). Positive staining of the lipid domains is consistent with known binding of uranyl ions to polar phospholipid headgroups (19).

Cytochrome *c* has a molecular weight of ~12,000; crystallographic data indicate that the holocytochrome is an oblate spheroid with principle axes larger than 3 nm (20). Based on these structural parameters, simple predictions may be made regarding expected changes in image averages of uranyl-stained VDAC arrays if the cytochrome were to bind to specific sites before negative staining with uranyl acetate. For example, if cytochrome *c* were to occupy the channels or tightly block their openings, the result should be a marked decrease in the stain density inside the channels. If it were to bind at or near (but not block) the channel openings, there would be a modulation of the stain distribution on the rims of the channels (i.e., in the region immediately adjacent to the dense stain sites). If the cytochrome were to bind at the lipid domains, the phospholipid headgroups should be less accessible to the uranyl ions, resulting in stain exclusion from this region. Finally, if the cytochrome were instead to fall at random on the array (i.e., with no specificity), there would be no net change in the final image average, i.e., stain exclusion due to random positioning of the cytochrome would be "averaged out" by the image analysis techniques to be used (Fourier and correlation averaging, see Materials and Methods).

MATERIALS AND METHODS

Preparation of Crystalline Arrays of Mitochondrial Pore Protein

Outer membranes were isolated from mitochondria of the fungus *N. crassa* (FGSC 326) by hypoosmotic lysis and sucrose step-gradient centrifugation as described previously (13). Crystallization of the pore protein in the plane of these membranes was carried out by the phospholipase A_2/dialysis technique first described by Mannella (21). Sucrose-gradient fractions containing freshly isolated mitochondrial outer membranes (~10 μg protein/ml) were pooled and divided into three 7-ml aliquots, to which were added, respectively, 0, 2.5, and 10 U of bee-venom phospholipase A_2 (Sigma Chemical Co., St. Louis, MO). These membrane suspensions were then dialyzed overnight in the cold against 200 vol of low-salt buffer (1 mM Tris-HCl, pH 7.5; 0.25 mM EDTA). After dialysis, the membranes were centrifuged (100,000 *g*, 60 min) and resuspended in 100 μl of 10 mM Tris-HCl, pH 7.5. Each membrane suspension was examined for content of crystalline membranes by negative stain electron microscopy (see below). The suspension not treated with phospholipase A_2 was found to contain very few array-bearing membranes (<10%). In both of the phospholipase-treated suspensions, the concentration of total membranes was about the same as that of the untreated sample but more than half of the membranes consisted all or in part of the characteristic crystalline arrays of VDAC. The suspension treated with the lower level of phospholipase A_2 was used in the subsequent cytochrome *c* binding experiments.

Specimen Preparation

15-μl aliquots of the mitochondrial outer membrane suspension containing crystalline VDAC arrays were mixed with 15 μL of 10 mM Tris-HCl (pH 7.25) containing (*a*) nothing else, (*b*) 1 mM apocytochrome *c* (prepared from beef heart holocytochrome *c*, gift of G. Shore, McGill

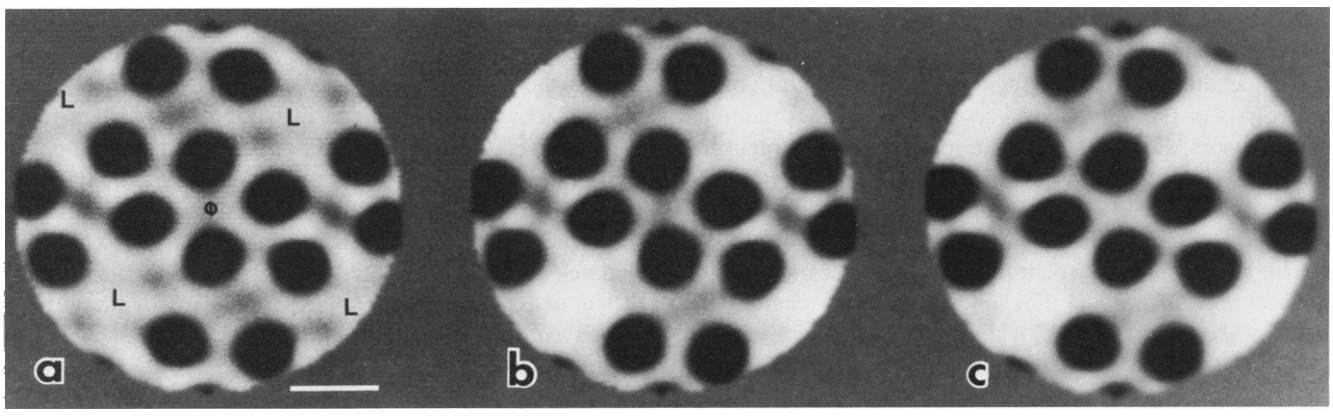

FIGURE 1 Electron microscopic images of negatively stained outer mitochondrial membrane channel arrays. Computed averages of (*a*) untreated membrane arrays, (*b*) arrays pre-incubated with apocytochrome *c*, (*c*) arrays pre-incubated with holocytochrome *c*. In the average images, dark regions indicate the presence of stain. In *a* the diad axis at the center of the six-channel cluster is indicated. *L*, lipid regions at the corners of the unit cell Bar, 5 nm.

University, Montreal), or (*c*) 1 mM holocytochrome *c* (beef heart, Sigma Chemical Co.). (The VDAC concentration in these specimens was ~1 μM so that putative receptor sites for apocytochrome *c* with an affinity constant of 10^7 M^{-1} would be expected to be saturated under these conditions.) After incubating for 15 min at room temperature, the mixtures were deposited in 5-μl aliquots on electron microscopic specimen grids (freshly glow-discharged, carbon/formvar-coated, 400 mesh Cu grids). After 1 min, excess liquid was blotted from the grids and the specimens were fixed with 10 μl of 1% glutaraldehyde for 1 min. The grids were then rinsed twice with 10 μl of 10 mM Tris-HCl (pH 7.25) and negatively stained with 10 μl of 1% uranyl acetate. The specimens were allowed to air dry and were examined in the electron microscope within a few hours.

Electron Microscopy and Image Processing

Electron micrographs were recorded in an electron microscope (model EM 301; Philips Electronic Instruments, Inc., West Nyack, NY) operated at 100 kV, using SO163 film (Eastman Kodak Co., Rochester, NY). Several dozen images were recorded from different grids of each of the three specimens, at a nominal magnification of 45,000. Fields were selected that contained one or more obviously crystalline membranes. Images to be processed further were selected from these micrographs by optical diffraction, using the criteria described by Mannella and Frank (17): (*a*) the crystalline regions were evenly stained and at least 0.25 μm^2 in area; (*b*) the optical diffraction patterns showed at least three strong orders of diffraction; (*c*) in the usual case of collapsed membrane vesicles, the reflections from the two sides did not overlap; and (*d*) the reciprocal lattices were oblique, with lattice angles in the range 106.5–110.5°.

The periodic array images selected by these criteria were digitized with a PDS 1010A flatbed scanning microdensitometer (Perkin-Elmer Corp., Garden Grove, CA). The scanning grid used corresponded to a sampling distance of 0.56 nm.

Averages were made of the different crystalline array images using the SPIDER image processing system (22), implemented on a VAX 11/780 computer (Digital Equipment Corp., Maynard, MA). The image averaging procedure employed in this study has been described in detail elsewhere (15). The first step involves quasi-optical Fourier filtration of a part of the crystalline image (23, 17). This basically entails filtering the Fourier transform of a field (which can vary from 180 × 180 to 256 × 256 square pixels) with a mask defined by the reciprocal lattice of one array layer, followed by inverse transformation of the passed Fourier components. The resulting Fourier average is then used as a reference to compute a correlation average of the entire crystalline field (24). The technique of correlation averaging overcomes limitations that may be imposed on the resolution of Fourier averages by lattice disorder (25, 26). An area of 44 × 44 square pixels centered on one cluster of six channels is windowed from the center of the Fourier-filtered field, padded into a 512 × 512 matrix, and cross-correlated with a 512 × 512 field in the unfiltered image. The resulting cross-correlation function contains peaks at locations in the raw image where there is a strong match with the reference image. A peak-search program generates a file containing the precise coordinates of the cross-correlation maxima. Spurious (obviously off-lattice) maxima are deleted from the peak file by visual inspection of cross-correlation maps produced with the peak file. The final image average is formed by addition of 64 × 64 areas windowed from the raw image at the coordinates specified by the peak file. Using this procedure, five image averages were successfully computed for the control (no cytochrome *c*) specimen, six for the + apocytochrome *c* specimen, and seven for the + holocytochrome *c* specimen.

These individual averages were next aligned rotationally by autocorrelation analysis (27) after twofold bilinear interpolation (to increase the accuracy of alignments) and magnification correction based on lattice parameters (17). Each average was then aligned translationally by centering on the twofold symmetry axis at the middle of the six-channel cluster. At this point, the consistency of the different image averages was assessed by calculating the cumulative phase residual of each image relative to every other (28), at a Fourier cutoff of 1/(2.5 nm). Three image averages showed phase residuals >45° with most of the other images and were discarded. These included two of the six + apocytochrome *c* set and one of the seven + holocytochrome *c* set. Final specimen averages were then calculated by summing the remaining averages in each set, after each image average had first been scaled to unit mean density. The variance at each point in the three specimen averages was simultaneously calculated and used to compute a map of the standard error of the mean associated with each image average (18). The total numbers of unit cells contributing to formation of each specimen average were: control, 1169; + apocytochrome *c*, 1081; + holocytochrome *c*, 1329. Each specimen average was then low-pass filtered at 1/(2.5 nm) in Fourier space and p2 symmetry was imposed (by summing each average with itself after 180° rotation about the central diad axis). After scaling each specimen average to unit mean density (18), the following "difference images" were calculated by subtraction: + apocytochrome *c* minus control, + holocytochrome *c* minus control, and + apocytochrome *c* minus + holocytochrome *c*. (Note, optimum scaling of the specimen averages used to form the difference images was checked by the criterion in reference 18, i.e., minimization of the overall difference between each pair of averages, in terms of the variance in the respective difference image. In each case, this difference was found to be minimized when the two images were scaled within 5–10% of each other, so scaling to common mean density is valid for this data set.)

RESULTS

As described in Materials and Methods, micrographs were recorded from uranyl-embedded arrays of the pore protein VDAC that had been preincubated with apo- and holocytochrome *c* under conditions like those used by Hennig et al. (7). Before negative staining, the specimens were fixed with glutaraldehyde under conditions (1% glutaraldehyde, 1 min, room temperature) that were known to effectively cross-link the polypeptides in isolated mitochondrial outer membranes without altering the projection images of VDAC arrays (Mannella, C. A., unpublished observations). This mild fixation was employed to prevent possible detachment of the cytochromes from protein receptors during subsequent negative staining. Average projection images (calculated as described in Materials and Methods) of VDAC arrays in the absence of cytochrome *c* and preincubated with the apo- and holocytochrome are presented as Fig. 1, *a–c*, respectively.

The control (no cytochrome *c*) image (Fig. 1 *a*) shows the central cluster of six densely stained channels and the lightly stained lipid domains at each corner of the parallelogram unit cell. In both the + apocytochrome *c* and + holocytochrome *c* images (Fig. 1, *b* and *c*), there is no significant decrease in stain accumulation at or in the immediate vicinity of the channel openings. The failure of the cytochromes to cause stain exclusion from the protein domains in negatively stained VDAC arrays argues against the binding to the pore-protein of either form of cytochrome *c*. However, there is a noticeable difference between the control and + cytochrome *c* images away from the channel protein, namely decreased uranyl staining in the lipid domains in the latter images.

Difference images were formed from the image averages in Fig. 1, *a–c* to better localize the regions of significant difference between the + cytochrome images and the

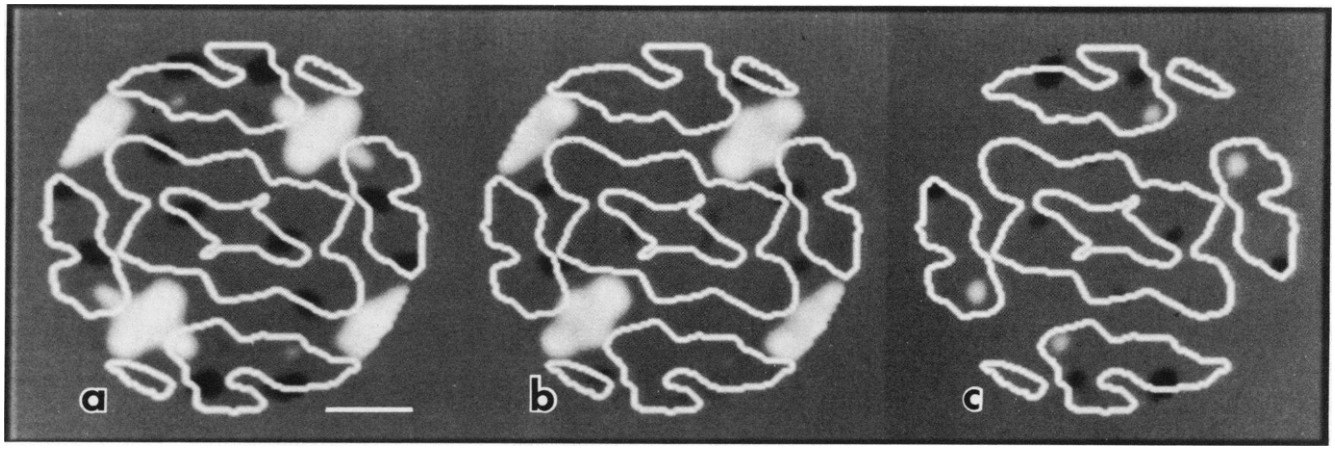

FIGURE 2 Difference images computed using the average images in Fig. 1, *a–c*. (*a*) + apocytochrome c minus control; (*b*) + holocytochrome c minus control; and (*c*) + apocytochrome c minus + holocytochrome c. The contours indicate the projected outline of the channel protein in the arrays. White regions indicate relative stain exclusion from the first image in each pair. Only differences greater than three-times the maximum standard error of the mean in each set are shown. Bar, 5nm.

control image, as well as between the + apocytochrome *c* and + holocytochrome *c* images. These difference images are presented in Fig. 2, superimposed on contour maps delineating the protein and lipid regions of the VDAC array, based on the density map of unstained crystalline VDAC (15). The difference images in Fig. 2 have been threshholded so that only differences greater than three-times the maximum standard error of the mean for the composite images are shown (18). White regions in the difference images indicate regions of stain exclusion in the first image relative to the second. Fig. 2, *a* and *b* clearly indicate that the presence of either apo- or holocytochrome *c* causes statistically significant stain exclusion from the lipid region of the VDAC arrays. There is no significant difference between the + apocytochrome *c* and + holocytochrome *c* images.

DISCUSSION

Using image processing of negative-stain electron micrographs it appears that the apo- and holo- forms of cytochrome *c* bind to the lipid domains in the crystalline channel arrays in outer membranes of *Neurospora* mitochondria. The same experiments provide no evidence for binding of either form of the cytochrome to the pore-protein itself.

Several objections may be raised to these experiments, in particular, the use of animal (and not fungal) cytochromes. However, the results of Hennig et al. (7) indicate that animal apocytochrome *c* competes with the *Neurospora* cytochrome for the high affinity binding sites on the *Neurospora* mitochondrial outer membrane. Other objections may be based on the techniques used to prepare the specimens for electron microscopy. It is unlikely that the negative stain (uranyl acetate) interfered with possible interactions between VDAC and cytochrome *c* since the specimens were chemically cross-linked before negative staining. Similarly, it seems unlikely that mild glutaraldehyde treatment itself somehow reversed putative VDAC-cytochrome *c* binding. Results essentially the same as those of Fig. 2 have been obtained without prior fixation using both uranyl acetate and phosphotungstate as negative stains (Mannella, C. A., and J. Frank, unpublished results).

It is important to note, however, that failure to detect alterations in stain distribution in the protein regions of crystalline VDAC arrays attributable to cytochrome *c* does not, strictly speaking, rule out the possibility of interactions between the two proteins. It may be, for example, that the VDAC protein in the two-dimensional crystalline arrays is in a nonphysiological conformation, one in which putative apocytochrome *c* binding sites might be inaccessible. While this possibility cannot be excluded at present, it seems unlikely for two reasons: (*a*) previous image analysis of the *Neurospora* VDAC arrays suggests that the protein is in a conformation that defines a channel whose size is consistent with the permeability properties of the native outer mitochondrial membrane (15); (*b*) antibodies raised against the native outer mitochondrial membrane, which are specific for the 31-kD pore protein and inhibit in vitro channel formation, bind to the crystalline arrays (14). It is also possible that bound apocytochrome *c* might not alter the stain distribution in the vicinity of the channels as much as expected based on its molecular size in solution. This might be the case if, for example, the cytochrome were to completely unfold upon binding to the channels. Thus, at present, we can only conclude that these experiments argue against (or at least provide no evidence in support of) VDAC acting as a receptor for apocytochrome *c*.

The localization of both apo- and holocytochrome *c* to the lipid regions in electron images of the pore protein

arrays may be associated with the "low affinity" binding sites for cytochrome *c* detected by Hennig et al. (7) on the outer surface of *Neurospora* mitochondria. In fact, the association constant of these low-affinity sites, 4×10^6 M^{-1}, is very close to that calculated for binding of ferricytochrome *c* to azolectin vesicles in 20 mM phosphate buffer at neutral pH, 2.2×10^6 M^{-1} (29). That the apocytochrome inserts into (and apparently penetrates) azolectin vesicles has been demonstrated by Dumont and Richards (30).

The affinity of apocytochrome *c* for phospholipids, indicated by our own and earlier studies, poses a serious problem in the search for possible protein receptors for the cytochrome on the outer mitochondrial surface. In the case of *Neurospora* mitochondria, much of the phospholipid in the outer membrane is exposed to the aqueous medium, accessible to soluble phospholipase A_2 (21). Thus, if there are low concentrations of high-affinity receptors on mitochondrial outer membranes, their presence may be masked by binding of apocytochrome *c* to phospholipid, especially in experiments done at low ionic strength (such as those of Hennig et al. [7]). In the case of ferricytochrome *c*, the affinity constant for azolectin varies inversely with ionic strength, extrapolating to 10^9 M^{-1} in water (29).

In light of the lipophilicity of apocytochrome *c*, the question may be raised whether, in fact, specific outer-membrane protein receptors are required to explain the available data concerning its targeting and uptake into mitochondria. The strongest argument in favor of specific protein receptors is the sensitivity of binding of apocytochrome *c* (and other import polypeptides) to mild proteolysis of the outer mitochondrial surface. It is also difficult to explain the apparent specificity of this binding without thinking in terms of protein receptors. Consider, however, that there is much experimental data that membrane proteins influence the organization of the lipid constituents of synthetic and biological membranes (31–33). Could specific groups or clusters of phospholipids exist in the native mitochondrial outer membrane, separated from the bulk lipid phase by virtue of interactions with specific membrane proteins, in much the same way that the lipid domains are formed in the crystalline VDAC arrays? Assuming such lipid clusters exist, might they vary in such characteristics as size or content of particular polar headgroups, conferring on them higher affinities for certain polypeptides (or classes of polypeptides) than for others? (The regular patches of lipid in the VDAC arrays are not likely receptor candidates since they do not show the required specificity, i.e, they bind both apo- and holoforms of cytochrome *c*.) Proteolytic alterations to the membrane proteins that "organize" these lipid clusters could lead to dispersal of the lipids and subsequent loss of "receptor" characteristics. The hypothesis that particular groups or classes of lipids may function as receptors for import polypeptides on the outer mitochondrial membrane is readily testable. In particular, it would predict loss of polypeptide binding to mitochondria after phospholipid headgroup modification, e.g., by hydrolysis with phospholipases *C* or *D*. Finally, a precedent may already exist for such "lipid receptors," on the mitochondrial inner membrane. Cardiolipin, a phospholipid that is almost entirely restricted to this membrane, has been implicated as the primary receptor for the enzyme phosphocreatine kinase (34).

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